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Short Communication

Sequence variants of the sarco(endo)plasmic reticulum Ca^{2+}-transport ATPase 3 gene *(SERCA3)* in Caucasian Type II diabetic patients (UK Prospective Diabetes Study 48)

A. Varadi[1], **L. Lebel**[2], **Y. Hashim**[3], **Z. Mehta**[3], **S.J.H. Ashcroft**[2], **R. Turner**[3]

[1] School of Biochemistry and Genetics, University of Newcastle upon Tyne, UK
[2] Nuffield Department of Clinical Biochemistry, University of Oxford, UK
[3] UK Prospective Diabetes Study Group, Diabetes Research Laboratories, University of Oxford, UK

Abstract

Aims/hypothesis. Type II (non-insulin-dependent) diabetes mellitus is a common heterogeneous metabolic disorder of largely unknown genetic aetiology. The sarco(endo)plasmic reticulum Ca^{2+}-transport ATPase (SERCA) plays an important part in the glucose-activated beta-cell Ca^{2+} signalling that regulates insulin secretion. Impaired function and expression of SERCA have been shown in islets of Langerhans from diabetic animal models and have also been associated with beta-cell apoptosis. Thus, the *SERCA3* encoding gene is a plausible candidate for a primary pancreatic beta-cell defect.
Methods. In this study, the entire coding and the promoter regions of *SERCA3* gene were screened by single-strand conformation polymorphism analysis in white Caucasian Type II diabetic patients.
Results. We found four rare missense mutations [Exon 4: Gln_{108}→*His* (CAG→*CAT*), Exon 14: Val_{648} →*Met* (GTG→*ATG*) and Arg_{674}→*Cys* (CGC→*TGC*), and Exon 15: Ile_{753}→*Leu* (ATC→*CTC*)]. The patients with Gln_{108}→*His*, Val_{648}→*Met* and Arg_{674}→*Cys* mutations, which may affect the E1P-E2P transition of *SERCA3* during its enzyme cycle, had normal body weight with marked hyperglycaemia and beta-cell dysfunction. That is an unusual phenotype only found in 6% of the Type II diabetic patients recruited for the UK Prospective Diabetes Study. In addition, five silent polymorphisms, six intron variants and two polymorphisms in the 3' untranslated region of exon 22 were found with similar frequency in diabetic and control subjects.
Conclusion/interpretation. Our result suggests that in white Caucasians, the *SERCA3* locus possibly contributes to the genetic susceptibility to Type II diabetes [Diabetologia (1999) 42: 1240–1243].

Keywords Sarco(endo)plasmic reticulum Ca^{2+} ATPase, single-strand conformation polymorphism, sequence variants, Type II diabetes.

Genetic factors play an important part in the pathogenesis of Type II (non-insulin-dependent) diabetes mellitus, a heterogeneous disorder characterised by defects in insulin action as well as insulin secretion [1]. Investigations of candidate genes for Type II diabetes have led to the identification of polymorphisms of several genes. Major genetic causes of this disease, however, remain elusive.

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Corresponding author: Dr A. Varadi, School of Biochemistry and Genetics, University of Newcastle, The Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK
Abbreviations: SERCA, Sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SSCP, single-strand conformation polymorphism; MDE, mutation enhancement; nt, nucleotide; UKPDS, UK Prospective Diabetes Study.

Glucose-dependent sequestration of Ca^{2+} into endoplasmic reticulum and its subsequent release play an important part in the control of intracellular Ca^{2+} concentration, which regulates insulin secretion in pancreatic beta cells [2]. The active uptake of cytosolic Ca^{2+} into endoplasmic reticulum is mediated by sarco(endo)plasmic reticulum Ca^{2+}-transport ATPases (SERCAs) in which the hydrolysis of ATP is coupled to Ca^{2+} transport through formation of a phosphoenzyme intermediate. This intermediate exists in two different functional states, E1P and E2P, whose interconversion is essential to energy utilisation [3]. Two SERCA isoforms SERCA2b and SERCA3 have been identified in pancreatic beta cells and islets of Langerhans [4]. The human *SERCA3* gene has been recently characterised and localised to chromosome 17 [5, 6]. It contains 22 exons, and exon 21 can

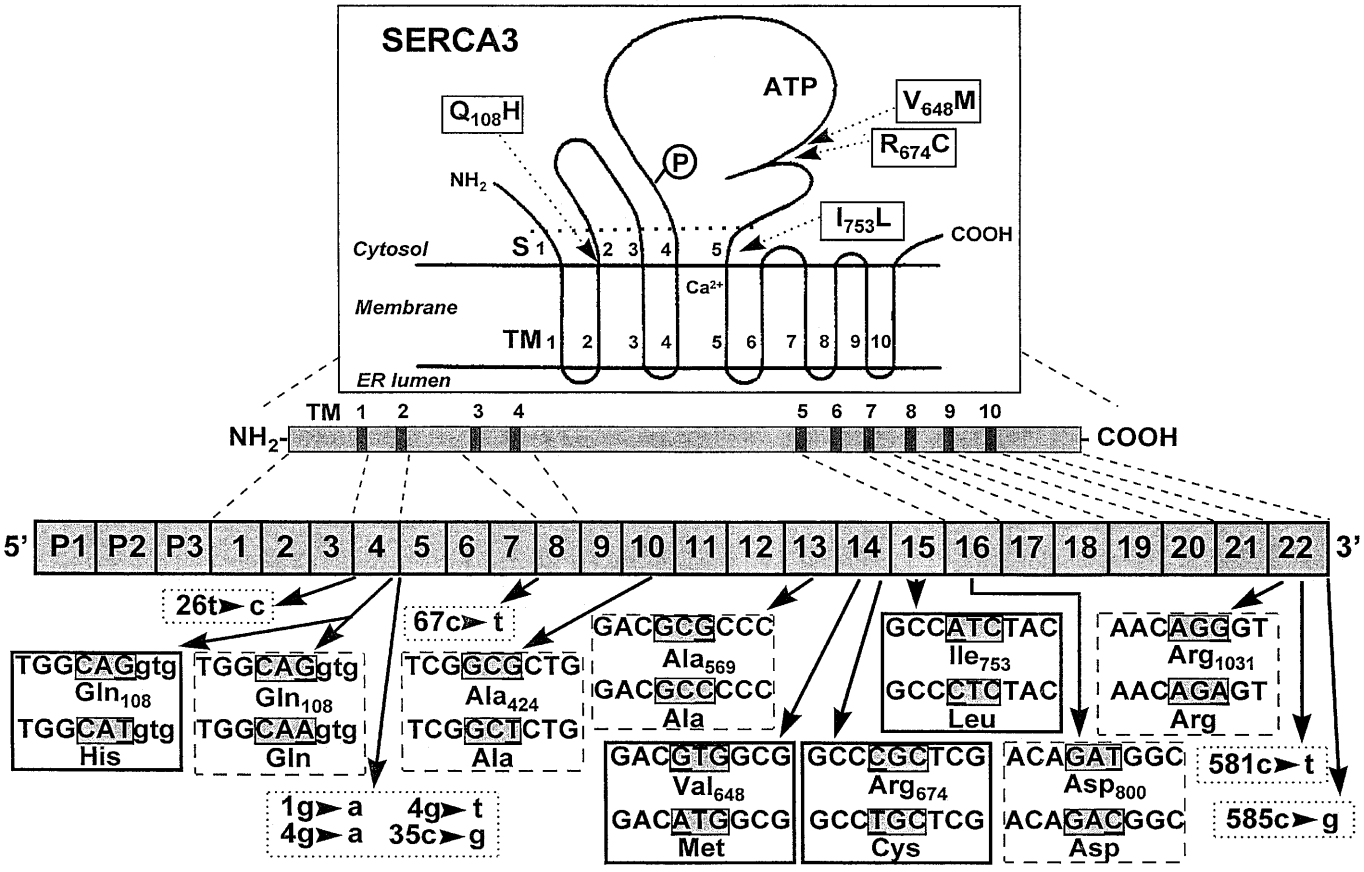

Fig. 1. Sequence variants in the human *SERCA3* gene. The top panel of the figure shows a structural model of sarco(endo)plasmic reticulum Ca^{2+}-ATPase protein. TM and S stand for transmembrane and stalk domains, respectively. The two Ca^{2+} binding sites are located in TM4, TM5, and TM6 membrane regions. The small and large cytoplasmic domains are located between TM2–3 and TM 4–5, respectively. The phosphorylation (P) and ATP binding (ATP) sites are in the large cytoplasmic domain. The positions of the four missense mutations found in this study are indicated in boxes using the one letter code for the amino acids. A schematic diagram of the SERCA3 protein with the ten transmembrane domains (TM1–10); and the *SERCA3* exons numbered from 1 to 22 and the 5′ non-coding region (P1-P3) is shown in the middle panel. The observed missense mutations (continuous line), silent mutations (dashed line) and sequence variants in the non-coding regions (dotted line) are indicated in boxes at the bottom of the figure. The sequence variants in the non-coding regions are labelled with lower case letters. The codons in which the variation occurs are highlighted and the affected bases are underlined

be excluded generating alternative spliced forms with different C-termini.

It has been proposed that abnormalities in Ca^{2+} signalling cause insulin resistance and impair insulin secretion and therefore possibly are a basic common pathology of Type II diabetes [7, 8]. Aberrant SERCA function was responsible for impaired glucose responses and defective insulin secretion in *db/db* mouse [8]. We have shown a significant reduction of SERCA3 expression in islets isolated from diabetic GK rats while SERCA2 expression was not significantly changed in the same sample [4]. Furthermore it was found that inhibition of SERCA activity caused apoptosis in MIN6 cells suggesting the critical importance of intracellular Ca^{2+} pools and SERCAs in maintaining the beta-cell viability [9]. Apoptosis mediates the beta-cell mass reduction observed in Type II diabetes.

In this study we have investigated whether *SERCA3* polymorphisms are associated with Type II diabetes. The entire coding region and the minimum promoter of *SERCA3* were screened by PCR and single-strand conformational polymorphism (SSCP).

Subjects and methods

Subjects. Sequence variants were identified by screening 88 white Caucasian Type II diabetic patients. We chose 63 non-obese subjects (body mass index < 25 kg/m^2), who had predominantly beta-cell dysfunction, from the UKPDS: 25 had mild diabetes with fasting plasma glucose < 10 mmol/l, 38 had fasting plasma glucose > 12 mmol/l. We randomly selected 25 newly diagnosed Type II diabetic patients and 25 non-diabetic control subjects. In addition, 97 random white Caucasian Type II diabetic and 75 non-diabetic control subjects were screened for the exon 4, 14 and 15 missense mutations, the exon 13 silent polymorphism and the intron 8 variants (Table 1.). The study was approved by the Oxford research ethics committee in accordance with the 1996 Declaration of Helsinki, and all subjects gave their informed consent.

SSCP analysis. Genomic DNA was extracted from peripheral blood using standard procedures. Primers (sequence available

Table 1. DNA polymorphisms in human *SERCA3* gene. The frequency of the seventeen polymorphisms identified in Type II diabetic and control subjects is listed in the appropriate columns. The number of subjects analysed by SSCP in each group is indicated by *n*. DNA polymorphisms found in introns are noted with respect to the splice donor or acceptor site (nt, nucleotide).

Location		Nucleotide change	Frequencies			
Exon	Codon		Normal subjects (*n*)	Random Type II diabetic subjects (*n*)	Beta-cell deficient Type II diabetic subjects (*n*)	Mild Type II diabetic subjects (*n*)
4	108	CAG(Gln)→CAT(His)	G1.00, T0.00 (100)	G1.00, T0.00 (122)	G0.97, T0.03 (38)	G1.00, T0.00 (25)
	108	CAG(Gln)→CAA(Gln)	G0.86, A0.14 (100)	G0.90, A0.10 (122)	G0.92, A0.08 (38)	G0.92, A0.08 (25)
10	424	GCG(Ala)→GCT(Ala)	G0.60, T0.40 (25)	G0.60, T0.40 (25)	G0.64, T0.36 (25)	G0.68, T0.32 (25)
13	569	GCG(Ala)→GCC(Ala)	G0.76, C0.24 (94)	G0.54, C0.46 (130)	G0.57, C0.43 (37)	N/A
14	648	GTG(Val)→ATG(Met)	G1.00, A0.00 (100)	G1.00, A0.00 (122)	G0.97, A0.03 (38)	G1.00, A0.00 (25)
	674	CGC(Arg)→TGC(Cys)	C1.00, T0.00 (100)	C1.00, T0.00 (122)	C0.97, T0.03 (38)	C1.00, T0.00 (25)
15	753	ATC(Ile)→CTC(Leu)	A1.00, C0.00 (100)	A0.99, C0.01 (122)	A1.00, C0.00 (38)	A1.00, C0.00 (25)
16	800	GAC(Asp)→GAT(Asp)	C0.72, T0.28 (25)	C0.68, T0.32 (25)	C0.72, T0.28 (25)	C0.68, T0.32 (25)
22	1031	AGG(Arg)→AGA(Arg)	G0.88, A0.12 (25)	G0.84, A0.16 (25)	G0.80, A0.20 (25)	G0.84, A0.16 (25)
	nt 581	C→T	C0.92, T0.08 (25)	C0.92, T0.08 (25)	C0.88, T0.12 (25)	C0.96, T0.04 (25)
	nt 585	C→G	C0.76, G0.24 (25)	C0.76, G0.24 (25)	C0.70, G0.30 (25)	C0.88, G0.12 (25)
Intron 4	nt 26	T→C	T0.76, C0.24 (25)	T0.72, C0.28 (25)	T0.60, C0.40 (25)	T0.64, C0.36 (25)
Intron 5	nt 1	G→A	G0.84, A0.16 (25)	G0.88, A0.12 (25)	G0.92, A0.08 (25)	G0.92, A0.08 (25)
	nt 4	G→A	G0.84, A0.16 (25)	G0.88, A0.12 (25)	G0.80, A0.20 (25)	G0.80, A0.20 (25)
	nt 4	G→T	G0.96, T0.04 (25)	G0.96, T0.04 (25)	G0.92, T0.08 (25)	G0.96, T0.04 (25)
	nt 35	C→G	C1.00, G0.00 (25)	C1.00, G0.00 (25)	C0.96, G0.04 (25)	C1.00, G0.00 (25)
Intron 8	nt 67	C→T	C0.95, T0.05 (100)	C0.94, T0.06 (122)	C087, T0.13 (38)	C0.92, T0.08 (25)

on request) were designed to amplify all 22 exons including the exon/intron boundaries and the 5' non-coding region of *SERCA3* [8] (European Molecular Biology Laboratory Accession numbers: Y15724–15730) from genomic DNA. PCR was carried out using standard reaction mixes containing 1.5–3.0 mmol/l $MgCl_2$, at 95 °C for 10 min, followed by 35–40 cycles at 95 °C for 1 min, 55–65 °C for 1 min and 72 °C for 1 min and a final extension step at 72 °C for 10 min. The PCR products were denatured and loaded onto 0.4x-1 x MDE (Flogen, Lichfield, Staffordshire, UK) gels and run at 160 V for 14–19 h at room temperature. After electrophoresis, gels were fixed in ethanol/acetic acid and stained using silver nitrate, following standard methods. Variants detected by SSCP were sequenced from both directions with a cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Warrington, UK). Differences between groups in genotypic frequencies were assessed by Fisher's exact test.

Results

SSCP analysis and nucleotide sequencing of the human SERCA3 gene. The SERCA3 coding region was divided into 33 (~ 300 bp) and the 5′ non-coding region into three (P1-P3) overlapping segments located between –609 and + 137 nucleotides containing the minimal promoter and the untranslated region (Fig. 1). In PCR amplification, the expected sizes of fragments of the *SERCA3* gene sequence were amplified. We detected 17 polymorphisms by SSCP analysis (Fig. 1). Sequencing of these PCR products showed that all of them resulted from a single nucleotide substitution corresponding to each of the SSCP polymorphisms. A substitution of an amino acid occurred in four of these polymorphismus: Gln_{108}→*His* (CAG→CAT), Val_{648}→*Met* (GTG→ATG), Arg_{674}→ *Cys* (CGC→TGC) and Ile_{753}→*Leu* (ATC→CTC). Each was identified in heterozygous form of one patient. In addition five coding polymorphisms: Gln_{108} (CAG→CAA), Ala_{424} (GCG→GCT), Ala_{569} (GCG→GCC), Asp_{800} (GAC→GAT), Arg_{1031} (AGG→AGA); six intronic changes intron 4 nt 26 T→C; intron 5 nt 1 G→A, nt 4G→A, nt 4 G→T, and nt 35C→G; and intron 8 nt 67C→T; and two sequence variants in the 3′ non-coding region of exon 22: nt 581 C→T and nt 585 C→G were identified. No sequence variant was found in the minimum promoter region or in the optional exon 21 or its 5′ donor and 3′ acceptor splice sites.

We found for ten sequence variants similar SSCP patterns in the 25 beta-cell deficient, 25 mild and 25 random Type II diabetic patients and in the 25 control subjects, and there was no significant difference in their frequency between diabetic and non-diabetic control subjects (Table 1.). For the missense mutations we screened a further 97 random Type II diabetic and 75 control subjects and no further patients with these variants were discovered (Table 1.). The allelic frequencies of the exon 13 silent variant differed between unselected Type II diabetic patients and control subjects (C allele frequency 46 % vs 24 %, respectively; $p < 0.0001$, Table 1) but this may be a type I error given the number of polymorphisms examined.

The three patients with exon 4 or 14 mutations had normal body weight with marked hyperglycaemia and beta-cell dysfunction, a phenotype only found in 6 % of the Type II diabetic patients recruited to UKPDS. Each had a family history of diabetes. The

patients with the exon 4 mutation developed severe, insulin requiring diabetes, whereas the two with exon 14 mutation responded unusually well to therapy with maintenance of normal fasting plasma glucose concentrations with diet and sulphonylurea respectively over 6 years follow-up. The patient with the exon 15 mutation was slightly overweight and, when allocated at random to UKPDS and insulin therapy, had good glucose control with modest insulin doses.

Discussion

The molecular mechanisms underlying the development of the common forms of Type II diabetes remain largely unknown. In this study the entire coding region and the promoter sequence of the *SERCA3* gene was analysed by SSCP in white Caucasian Type II diabetic patients to identify polymorphisms and assess their possible contribution to the development of Type II diabetes.

We have identified four rare missense mutations – $Gln_{108}\rightarrow His$, $Val_{648}\rightarrow Met$, $Arg_{674}\rightarrow Cys$ $Ile_{753}\rightarrow Leu$ – in Type II diabetic patients. These mutations affect residues that are conserved in human [5, 6] SERCA3 and in the structurally related human [10] SERCA1 protein. Site-directed mutagenesis has been extensively used to explore relations of structure and function in SERCA1 [3]. These studies showed that mutations blocking the crucial E1P to E2P transition are located in the small and the large cytoplasmic domains as well as in transmembrane segments M4 and M8. During the reaction cycle of the Ca^{2+}-ATPase the E1P phosphoenzyme intermediate normally loses its ADP sensitivity and its occluded Ca^{2+} within a few hundred milliseconds. Mutations to the residues that are crucial for the E1P to E2P transition lead to accumulation of the phosphoenzyme in the ADP-sensitive form. It has been suggested that in these mutants the Ca^{2+} is possibly retained in the occluded state for an unusually long time (no occurrence of translocation or opening of the luminal gate of the channel) [3]. The $Gln_{108}\rightarrow His$ mutation, which is located at the boundary of the second transmembrane and the small cytoplasmic domain, the $Val_{648}\rightarrow Met$ and $Arg_{674}\Leftrightarrow Cys$ mutations, which are located in the large cytoplasmic domain, could affect the E1P-E2P transition. These mutations were present in normal weight subjects with marked hyperglycaemia representing an unusual phenotype only found in 6% of the diabetic population. Each had a family history of diabetes. The patient with $Gln_{108}\rightarrow His$ mutation had progressive, beta-cell deficient diabetes requiring large doses of insulin whereas the patients with $Val_{648}\rightarrow Met$ and $Arg_{674}\rightarrow Cys$ mutations responded unusually well to diet alone or to sulphonylurea therapy, with normal glucose concentrations over 6 years of therapy.

The $Ile_{753}\rightarrow Leu$ mutation is located at the boundary of the M5 transmembrane domain. It has been shown that mutations at the M5 transmembrane and S5 stalk segment boundary lead to uncoupling of ATP hydrolysis from Ca^{2+} uptake. It has been suggested that uncoupling may be due to efflux through the Ca^{2+}-ATPase of Ca^{2+} that has been transported [3]. The $Ile_{753}\rightarrow Leu$ substitution could therefore affect the energy coupling of *SERCA3* leading to ATP hydrolysis without translocation of Ca^{2+}. The patient with $Ile_{753}\rightarrow Leu$ mutation was slightly overweight and had good glucose control on modest doses of insulin. Based on these observations, all four missense mutations identified in this study could potentially impair Ca^{2+} uptake via SERCA resulting in changed Ca^{2+} homeostasis which has been connected with the development of Type II diabetes [7, 8]. Moreover, the unusual phenotypes could be in accord with clinical expression of these missense mutations.

In this study, the minimum promoter region of *SERCA3* excluding the potential regulatory sites –6600 to –609 was analysed and no sequence variant was identified in diabetic and control subjects. Since decreased expression of SERCA3 mRNA was observed in diabetic GK rat [4] there is a possibility that polymorphisms in the more distal 5' regulatory sequence could be implicated in contributing to Type II diabetes.

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